Naval Medical Research Institute
8901 Wisconsin Avenue
Bethesda, MD 20889-5607

NMRI 95-79 November 1995

LIPID PEROXIDATION FOLLOWING *IN VITRO* AND *IN VIVO* O_2 EXPOSURES

A. L. Harabin
J. C. Braisted

Naval Medical Research
and Development Command
Bethesda, Maryland 20889-5606

Department of the Navy
Naval Medical Command
Washington, DC 20372-5210

NOTICES

The opinions and assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the naval service at large.

When U. S. Government drawings, specifications, or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from the Naval Medical Research Institute. Additional copies may be purchased from:

National Technical Information Service
5285 Port Royal Road
Springfield, Virginia 22161

Federal Government agencies and their contractors registered with the Defense Technical Information Center should direct requests for copies of this report to:

Defense Technical Information Center
Cameron Station
Alexandria, Virginia 22304-6145

TECHNICAL REVIEW AND APPROVAL

NMRI 95-79

The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

THOMAS J. CONTRERAS
CAPT, MSC, USN
Commanding Officer
Naval Medical Research Institute

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 1995	Technical 10/92 - 10/94

4. TITLE AND SUBTITLE
Lipid peroxidation following in vitro and in vivo O_2 exposures.

5. FUNDING NUMBERS
PE - 63713N
PR - M0099
TA - .01C
WU - 1011

6. AUTHOR(S)
Harabin, A.L. and J.C. Braisted

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Naval Medical Research Institute
Commanding Officer
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5607

8. PERFORMING ORGANIZATION REPORT NUMBER
NMRI 95-79

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Naval Medical Research and Development Command
National Naval Medical Center
Building 1, Tower 12
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5606

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
DN248526

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Four indices of lipid peroxidation were studied in rat brain, lung, and plasma to determine if these would be useful markers of progressive oxidative damage. Conjugated dienes, thiobarbituric acid reactive substances, and water and lipid soluble fluorescent products were measured following *in vitro* and *in vivo* O_2 exposures. For *in vitro* studies, homogenized tissues were oxidized non-enzymatically in increasing amounts of O_2. For *in vivo* studies, rats were exposed to normobaric or hyperbaric O_2 for a range of times and pressures. *In vitro*, all 4 indices increased with PO_2 of exposure: brain > lung > plasma. *In vivo* exposures resulted in lower levels than those achieved *in vitro*. Hyperbaric exposure produced higher levels than normobaric exposures, but fairly long duration and/or high PO_2 exposures were required for increases in tissues. On the other hand, plasma thiobarbituric acid reactive substances increased after only 6 h of normobaric O_2 exposure and remained elevated. Brain lipid peroxidation was detected following normobaric O_2, giving evidence of non-pulmonary oxidation. These results show that there is a relationship between severity of O_2 exposure and level of lipid peroxidation, but suggest that other markers should also be investigated for earlier changes.

14. SUBJECT TERMS	15. NUMBER OF PAGES
hyperbaric oxygen toxicity, central nervous system oxygen toxicity, pulmonary oxygen toxicity, thiobarbituric acid reactive substances, conjugated dienes, fluorescent products	31
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

The authors are especially grateful to Ms. Tracy Cope and Mr. Roberto Prescod for excellent technical assistance, to Ms. Cope for preparation of figures, and Ms. Susan Mannix and Ms. Janet Gaines for preparation of this manuscript.

This work was supported by the Naval Medical Research and Development Command Work Unit No. 63713N M0099.01C-1011. The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, Publication No. 85-23.

INTRODUCTION

This work was designed to determine whether indices of lipid peroxidation would increase in a dose-dependent way following normobaric and hyperbaric O_2 exposure. Lipid peroxidation, perhaps caused by free radicals, is often cited as one of the fundamental mechanisms of oxidative damage. Oxidation of lipids could lead to important changes in enzyme (1) or membrane (2) structure and function.

Free radical induced lipid peroxidation has been demonstrated repeatedly *in vitro* and the resultant cascade of events has been well described (3, 4) and is outlined in Fig. 1.

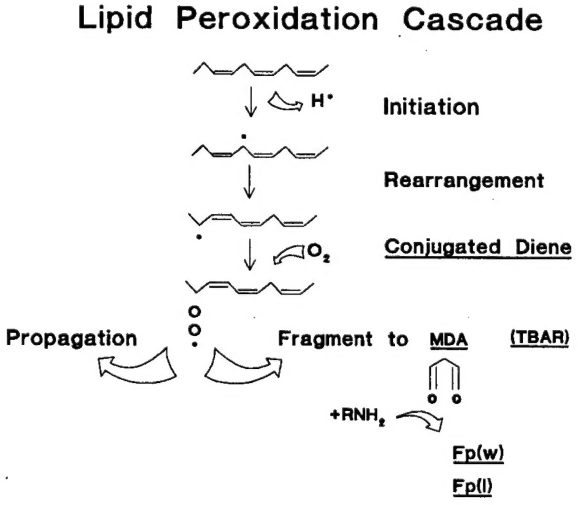

Figure 1. Lipid peroxidation cascade and chain reaction. A free radical can abstract a hydrogen atom from an unsaturated lipid molecule (initiation). The double bonds will rearrange to a more stable conjugated diene. An additional O_2 molecule can react with the relocated radical to form a lipid peroxy radical (R-OO•). Peroxy radicals can abstract a H• from another lipid molecule (propagation). If the lipid has ≥ 3 double bonds, it can fragment to form malondialdehyde (**MDA**). **MDA** is reactive and can form fluorescent products by cross-linking with amines. These products will be water (**Fp(w)**) or lipid (**Fp(l)**) soluble, depending on the nature of the amine.

Briefly, a hydrogen atom is abstracted from an unsaturated lipid molecule by a reactive radical. The double bonds will rearrange to form a more stable conjugated diene and O_2 will add to the relocated radical to form a lipid peroxy radical. The lipid peroxy radical can propagate the reaction by abstracting a H• from another lipid, or if the lipid species has 3 or more double bonds, it can fragment to form malondialdehyde. Malondialdehyde can react with amines (in nucleic acids, proteins, phospholipids etc.,) to form fluorescent chromophores (5). These fluorescent products can be lipid or water soluble, depending on the reactive amine. Each of the products along the lipid peroxidation cascade is unstable to some degree.

Our goal was to make measurements at several points along this lipid peroxidation cascade to determine which, if any, reflect the progression of damage from O_2. We began with *in vitro* exposures of isolated tissues and proceeded to whole animal studies in a range of O_2 levels. We included brain and lung tissue because these are thought to be primary targets of hyperbaric and normobaric O_2 toxicity, respectively. We also analyzed plasma to determine if changes were reflected in this easily accessible compartment.

We measured conjugated dienes (**CD**) both because these represent an early point along the lipid peroxidation cascade and these have been suggested to be a sensitive marker in other models of free-radical induced lung damage (6, 7). We also measured thiobarbituric acid reactive substances (**TBAR**), probably the most commonly measured marker of lipid peroxidation. Finally, we measured fluorescent products of both lipid (**Fp(l)**) and water (**Fp(w)**) soluble nature. The results show that markers of lipid peroxidation form following a range of O_2 exposures and that there is a relationship between severity of O_2 exposure and level of lipid peroxidation. In tissues, rather long duration or high PO_2 *in vivo* exposures

were required for changes and there was no obvious advantage to the alternatives to **TBAR**. Plasma analysis offered some promise of showing early changes from O_2 exposure.

METHODS

Animals

Adult virus-free male Sprague-Dawley rats (250-350 g) were used for all exposures.

In vitro studies

Brain, lung, and blood were obtained from rats for *in vitro* oxidation for initial investigation of the PO_2 dependence of lipid peroxide formation. Blood was centrifuged at 4 °C to obtain plasma. Brain cortex and lung tissue was removed, rinsed, diluted in buffer (50 mM KH_2PO_4, pH 7.4) and homogenized with a Polytron®. Control samples were taken and immediately placed in chloroform and methanol to stop any reactions or assayed immediately for **TBAR** as described below. Aliquots of tissue or plasma were then oxidized non-enzymatically (8) for 1 h by exposure to a final concentration of 10 μM $FeSO_4$ and 0.25mM l-ascorbate and humidified gases containing 5, 21, or 100% O_2 in a shaking water bath at 37 °C. Lung was diluted 1:5 and brain 1:45. Desferoxamine (1 mM) was added to all samples immediately prior to analysis to prevent further metal catalyzed breakdown of lipid peroxides. Samples were then immediately assayed for **CD**, **TBAR**, **Fp(l)**, and **Fp(w)**. Six experiments were completed and results were analyzed with a one-way analysis of variance (ANOVA) with repeated measures. Individual comparisons were made using Duncan's test when significant ($P < 0.05$) F values were obtained.

In vivo exposures

Rats were exposed to either hyperbaric or normobaric O_2 or served as controls as outlined in Table 1. There were at least 6 animals in each group. Normobaric exposures were conducted for 6, 24, 48 or 55^+ h. The 55^+ group receiving the longest exposure was

TABLE 1. Length of rat O_2 exposures conducted *in vivo*.

Group	Pressure (ATA)	Gas	Length (SD) (n animals)
Control	1	Air	- (10)
Normobaric	1	100% O_2	6 (7) 24 (9) 48 (10) 55^+ (12)
	1	85% O_2	5 days (8)
Hyperbaric -Short	5	100% O_2	47 (±3) min (6)
Long	5	100% O_2	83 (±28) min (6)
Short	6	100% O_2	20 (±4) min (6)
Long	6	100 O_2	47 (±8) min (6)

designed to be a nearly terminal exposure and therefore had some variability in its duration receiving an average of 56 ± 3.6 (SD) h. Hyperbaric exposures were to 5 or 6 atmospheres absolute (ATA) for either a short or a long duration. The short hyperbaric exposures were

chosen to be long enough to produce a convulsion, which is a definite symptom of hyperbaric O_2 toxicity. The long exposures were conducted until cessation of breathing. In all these exposures FI_{O2} was maintained at >98% and FI_{CO2} at < 0.05%. An additional group of animals was exposed to 85% O_2 at 1 ATA (normobaric) for 5 days. This exposure profile results in animals that show enhanced tolerance to a subsequent exposure to 100% O_2 (9). Results were analyzed using ANOVA and Duncan's test for individual comparisons where significant F values were obtained.

Tissue preparation

Following O_2 exposures, rats were immediately weighed and decapitated. Blood was collected in a heparinized tube, brains and lungs were quickly removed, rinsed with saline, and frozen in liquid N_2. Organs were weighed, homogenized in a cold buffer that contained 1mM desferoxamine, and immediately assayed for **CD**, **TBAR**, **Fp(l)**, and **Fp(w)**. Brains were diluted 1:6 (w/vol)). Lungs were diluted with 5 ml of buffer or were brought up to 7 ml total.

A control study was conducted to determine the need for the quick freezing procedure. In 4 control and 4 hyperbaric O_2 exposed animals, half of each organ was frozen while the other half was immediately homogenized. Both halves were then assayed. A paired t-test was used to compare results.

Biochemical assays

Thiobarbituric acid reactive substances (**TBAR**) were measured in tissues using a modification of a fluorescent assay described by Ohkawa et al. (10) using 1,1,4,4-tetramethxyopropane (TMP) as a standard. In addition to the 1 mM desferoxamine in the

homogenates, butylated hydroxytoluene was added (3) to a final concentration of 0.05% in the assay tubes to protect against further lipid peroxidation during the hot acidic conditions of the assay. The tubes were incubated for 1 h at 95 °C and extracted with *n*-butanol as described (10). Plasma was assayed according to Takeda et al. (11) where phosphotungstic was substituted for acetic acid. Recoveries of a known amount of TMP were measured in triplicate samples of each brain and lung assayed to account for quenching due to blood or other components of crude homogenates. Results corrected for **TMP** recovery were expressed as nmol/g brain wet weight, nmol/lung, and μM in plasma.

CD, **Fp(l)**, **Fp(w)** were measured following a lipid extraction by a modification of the technique of Bligh and Dyer (12). Five hundred μl of tissue homogenate were added to 7 ml of 2:1 chloroform and methanol. An additional 500 μl of buffer were added to bring the total aqueous phase to 1.0 ml. Tubes were vortexed for 2 min. Additional methanol was added (2.5 ml) and vortexed for another 2 min. The same volume of distilled water (acidified to pH 2.5 with HCl) was added to each tube and each was gently inverted, placed in an ice water bath to facilitate phase separation, and then centrifuged for 10 min at 1500 g. This procedure resulted in clear organic/aqueous separations and good recoveries (91%) of known amounts of lipid (corn oil). The water-methanol layer was aspirated for measurement of fluorescence for **Fp(w)**. Three milliliters of the chloroform layer were dried under a steam of N_2 that had been passed through a gas purifier and redissolved in 3 ml of heptane. **Fp(w)** and **Fp(l)** were measured by reading the fluorescence (excitation 360 nm; emission 443 nm) of the aqueous and heptane solutions respectively. **CD** was measured in the heptane by recording absorbance from 210 to 280 nm and reported as the peak absorbance at 233 nm. **CD**, **Fp(l)**, and (**Fp(w)**

were expressed as absorbance or fluorescence per lung or per gram brain tissue. Lipid concentration in the heptane layer was determined by the method of Chiang (13). Stability of the fluorimeter output was monitored daily with distilled H_2O or quinine sulfate. All samples were run in triplicate with appropriate blanks. Results are expressed as means ± 1 SD. In studies analyzed with ANOVA, the SD was obtained from the pooled estimate of the population's variance.

RESULTS

In vitro exposures

A 1-hour exposure to an *in vitro* oxidizing system consisting of iron, ascorbate and increasing amounts of O_2 raised all measured indices of lipid peroxidation in homogenized brain cortex and lung as shown in Figs. 2 and 3. All O_2 exposures increased values over unincubated controls, and values obtained after exposure to 100% O_2 were higher than after 21% O_2. Only brain **TBAR** and **Fp(l)** increased significantly between 5 and 21% O_2. Per gram of tissue, brain produced far more **TBAR** (80x control) and **Fp(l)** (10x control) than lung. In lung, most variables increased to a maximum of 2x control. In plasma (not shown), significant increases over control were found following exposure to 100% O_2 in **TBAR**, **CD**, and **Fp(w)**. The maximum increases were 8%, 6%, and 100% respectively over control.

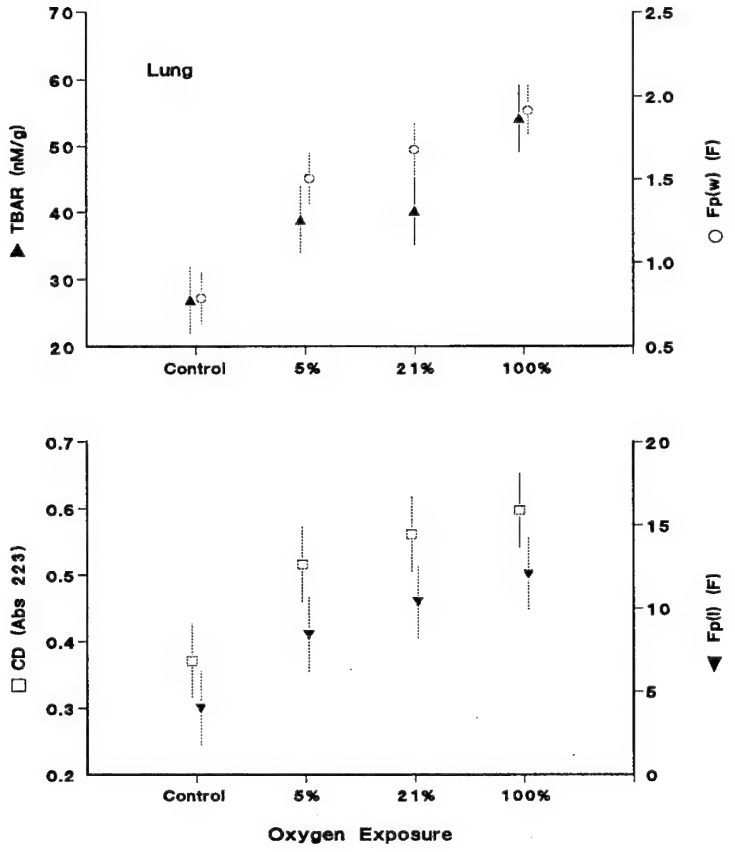

Figure 2. Effect of one hour *in vitro* oxidation in homogenized rat lung tissue: **TBAR** (thiobarbituric acid reactive substances), **CD** (conjugated dienes), **Fp(w)** (water soluble fluorescent products) **Fp(l)** lipid soluble fluorescent products) n=6, mean ± 1 SD. All O_2 exposures significantly increased all four variables over unincubated controls; samples exposed to 100 O_2% were higher than 5 and 21% exposed samples.

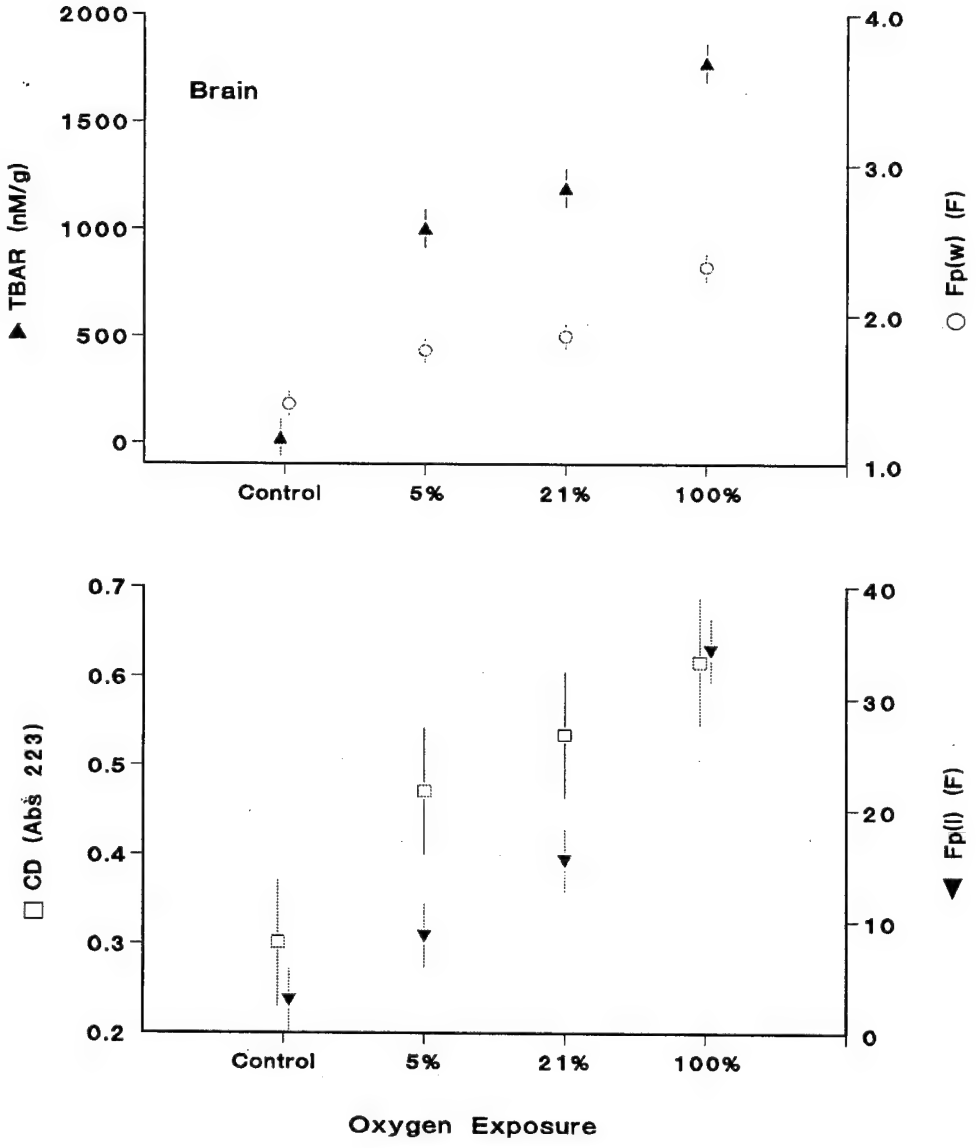

Figure 3. Effect of one hour *in vitro* oxidation in homogenized rat brain tissue. All O_2 exposed samples were increased over unincubated controls, and for **TBAR** and **Fp(l)** each increment in O_2 level produced an increment in lipid peroxide production. Abbreviations as in Fig 2.

In vivo exposures

Effect of freezing

Brain tissue that was not quickly frozen before assay had significantly lower **TBAR** (-14 ± 10%) and higher **Fp(w)** (+22 ± 8%). Other measurements were not significantly altered by freezing (with a 95% confidence interval averaging 27% of the mean control value). Although these differences were relatively small, all studies were carried out on quickly frozen tissues.

Physiologic effects of O_2 exposures

Both normobaric and hyperbaric O_2 exposures caused lung damage and pulmonary edema as judged by increases in lung weights as shown in Table 2. Forty-eight hours was the earliest time a significant increase was noted following O_2 at 1 ATA. Pleural fluid accumulation is a characteristic of pulmonary O_2 toxicity (in rats) and several milliliters of fluid were recovered following 48 and 55$^+$ h and 5-day 85% O_2 normobaric exposures. It is interesting that no fluid accumulated following the hyperbaric exposures (despite large increases in lung weight). We have reported previously that longer exposures to 2.8 ATA (14) also did not result in fluid accumulation. To account for this increased lung weight, results were expressed per organ. Brain weight did not change.

TABLE 2. Lung weight in grams following *in vivo* O_2 exposures. Numbers of animals in each group is given in Table 1.

	100% O_2 1 ATA (h)				85% (d)	5 ATA (min)		6 ATA (min)	
Control	6	24	48	55[+]	5	47	83	20	47
1.4	1.4	1.5	2.2[*]	2.2[*]	2.6[*]	3.4[*]	3.4[*]	4.2[*]	3.5[*]

*P≤0.05, (pooled SD of lung weights = 0.66 g)

Although the fluorescent assay for **TBAR** is less subject to interference, quenching occurred in the **TBAR** measurements in lung and brain following O_2. For lungs, recovery of a known amount of TMP changed from 85% in control lungs to 40% in O_2 exposed lungs (±9.4%). Recovery of TMP in brain decreased significantly by about 10%; recovery in controls was 100%. Each **TBAR** measurement was corrected for quenching by its own recovery of added **TMP** as an internal standard.

Symptoms of central nervous system O_2 toxicity resulted from the hyperbaric exposures. The short hyperbaric exposures were conducted until a convulsion developed. At 5 ATA the time required was 47 (±3) min; at 6 ATA, the required time was 16 (±4) min. The long exposures described in Table 1 were terminal; convulsions were observed in those groups after 36 (±11) min at 5 ATA and 24 (±3) min at 6 ATA.

Lipid peroxidation results

Figures 4 and 5 show the results for the 4 variables measured in lung. Figure 4 presents results of normobaric exposures and shows that increases in **CD** and **TBAR** were not evident until the very last day of survival (the 55^+ h time point). Animals exposed to 85% O_2 for 5 days showed increased lung **TBAR** and **CD**. Increases were noted following hyperbaric exposures as illustrated in Fig. 5. **TBAR**, **Fp(l)**, and **Fp(w)** all increased following long 5 ATA and short and long 6 ATA exposures. The high levels achieved following hyperbaric exposures were higher than those following normobaric ($p \leq 0.05$), although the levels obtained at 6 ATA were not higher than those at 5 ATA, and time had no significant influence (i.e., the long exposures were not significantly different than short ones).

Figures 6 and 7 show results for brain tissue. Figure 6 shows that **TBAR** and **Fp(w)** increased on the final day of survival with normobaric exposures. Hyperbaric exposures (Fig. 7) resulted in increased **TBAR** after short exposures (higher than the 55^+ normobaric value ($p \leq 0.05$)) and **Fp(l)** after long ones.

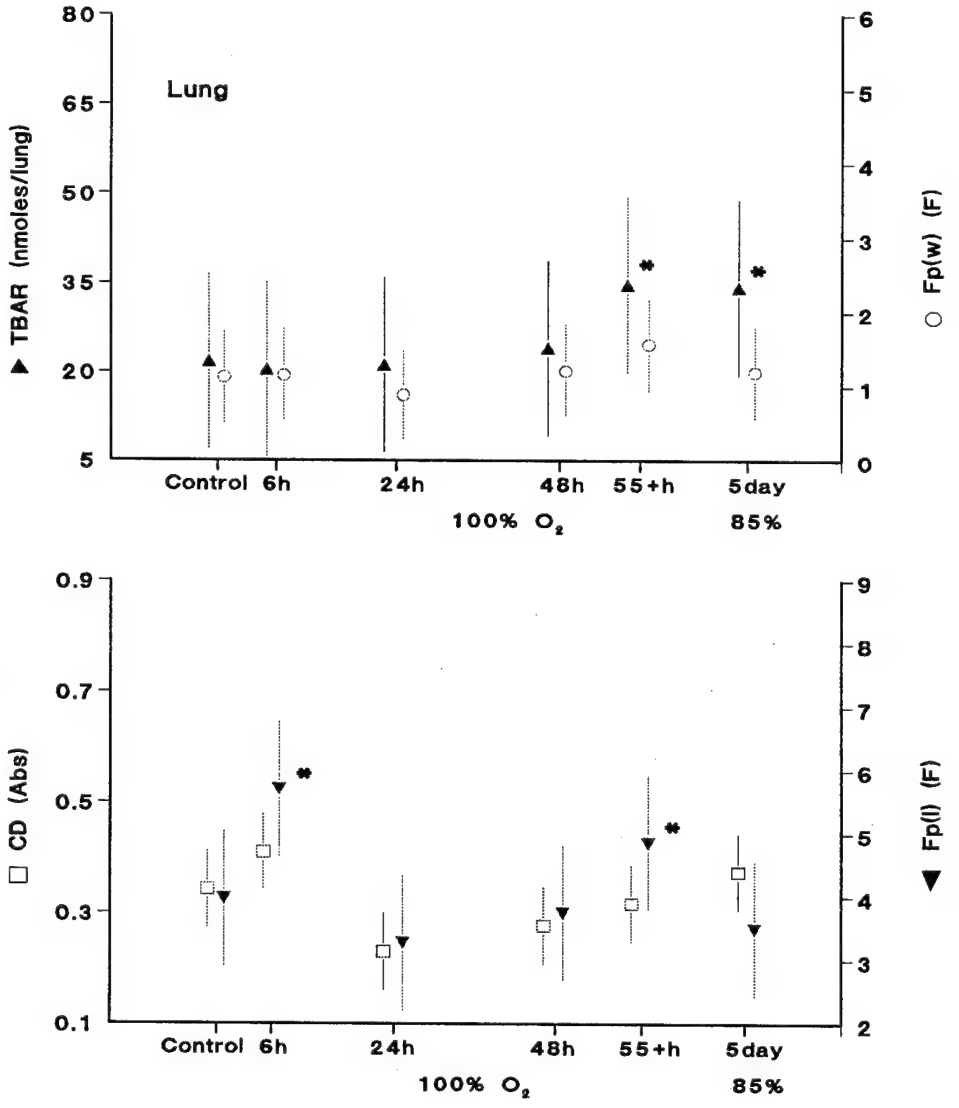

Figure 4. Effect of *in vivo* normobaric O_2 exposures on 4 indices of lipid peroxidation in lung. Rats were exposed to 100% O_2 for 6, 24, 48 or 55^+ h or to 85% O_2 for 5 days, at least 6 animals in each group. Results are means ± 1 SD. * Different from control, $P < 0.05$. Abbreviations as in Fig 2.

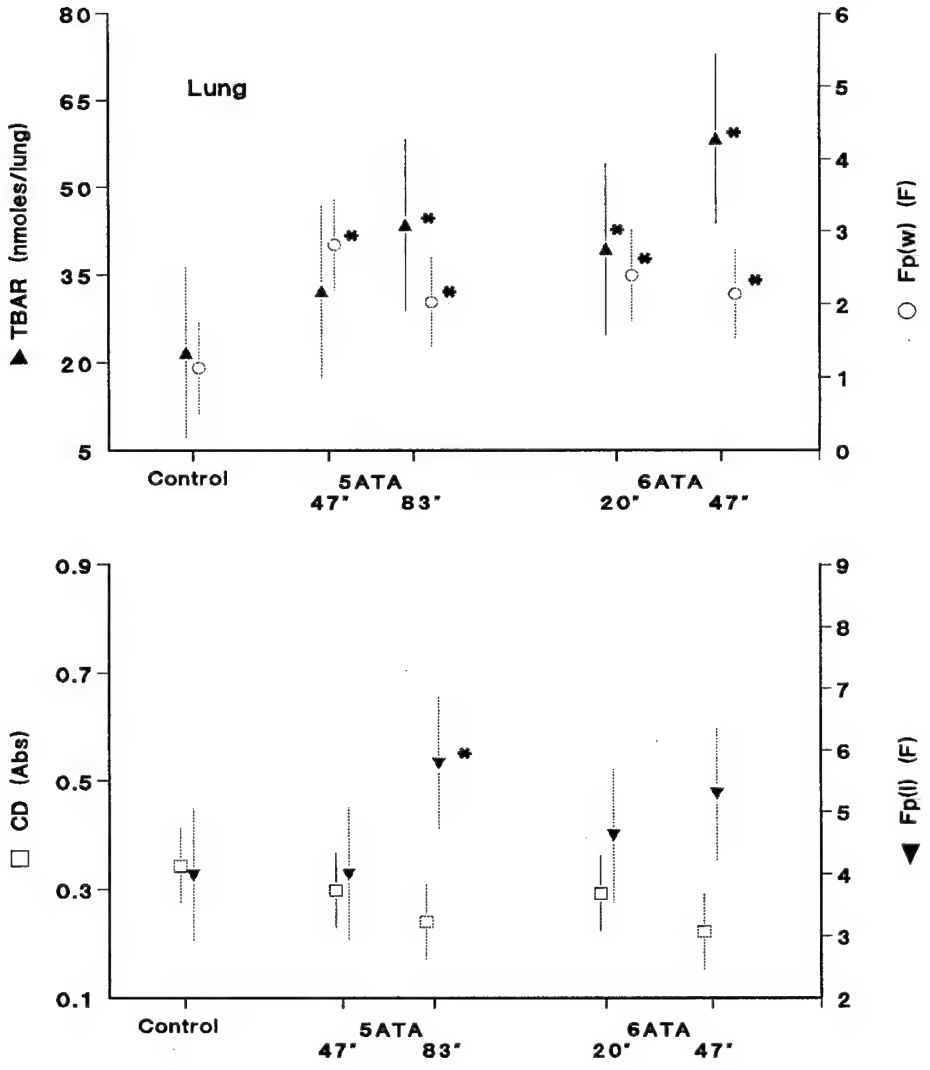

Figure 5. Effect of *in vivo* hyperbaric O_2 exposure on 4 indices of lipid peroxidation in rat lung. Animals were exposed to 5 or 6 ATA until development of a convulsion (shorter duration exposures) or until breathing ceased (longer duration). Results are from at least 6 animals in each group, mean ± 1 SD. * Different from control, $P < 0.05$. Abbreviations as in Fig. 2.

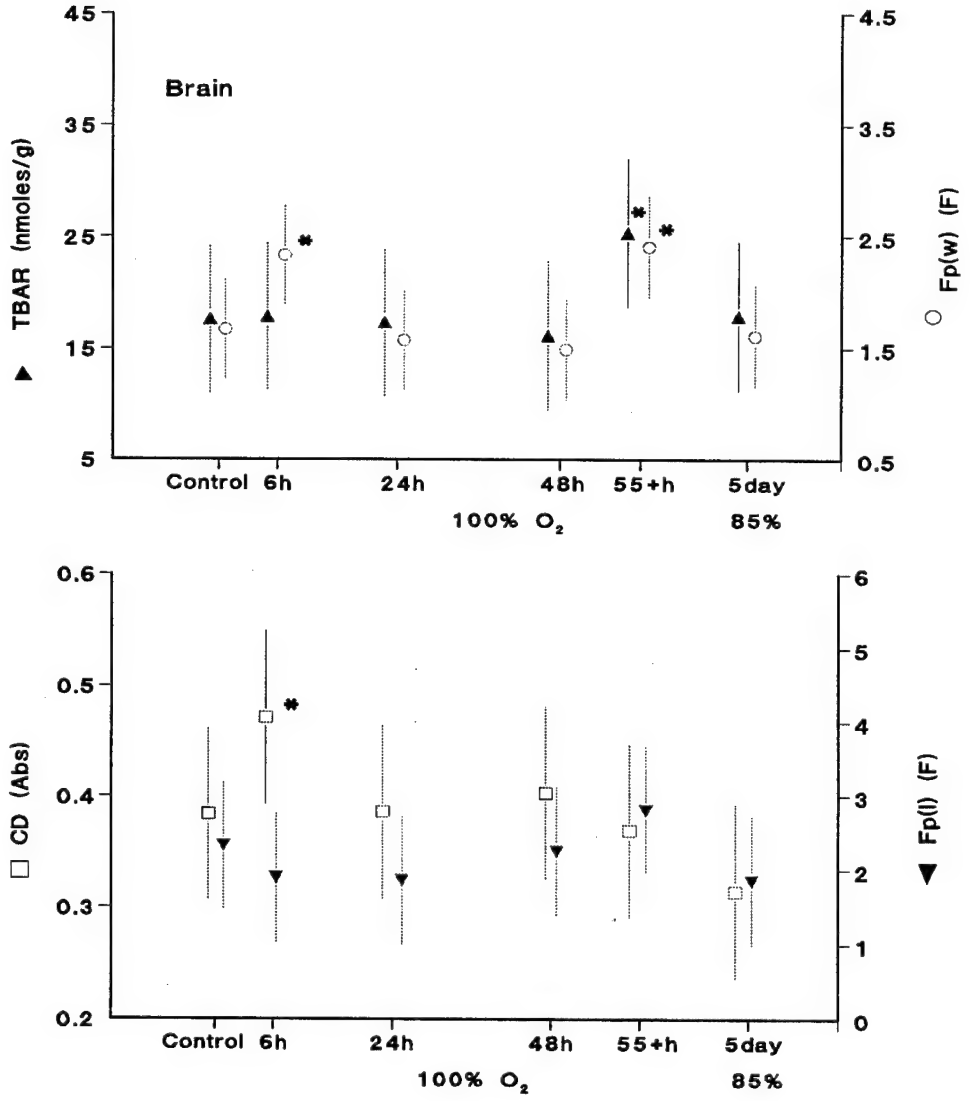

Figure 6. Effect of *in vivo* normobaric O_2 exposures on 4 indices of lipid peroxidation in brain. Rats were exposed to 100% O_2 for 6, 24, 48 or 55$^+$ h or to 85% O_2 for 5 days, at least 6 animals in each group. Results are means ± 1 SD. * Different from control, $P < 0.05$. Abbreviations as in Fig 2.

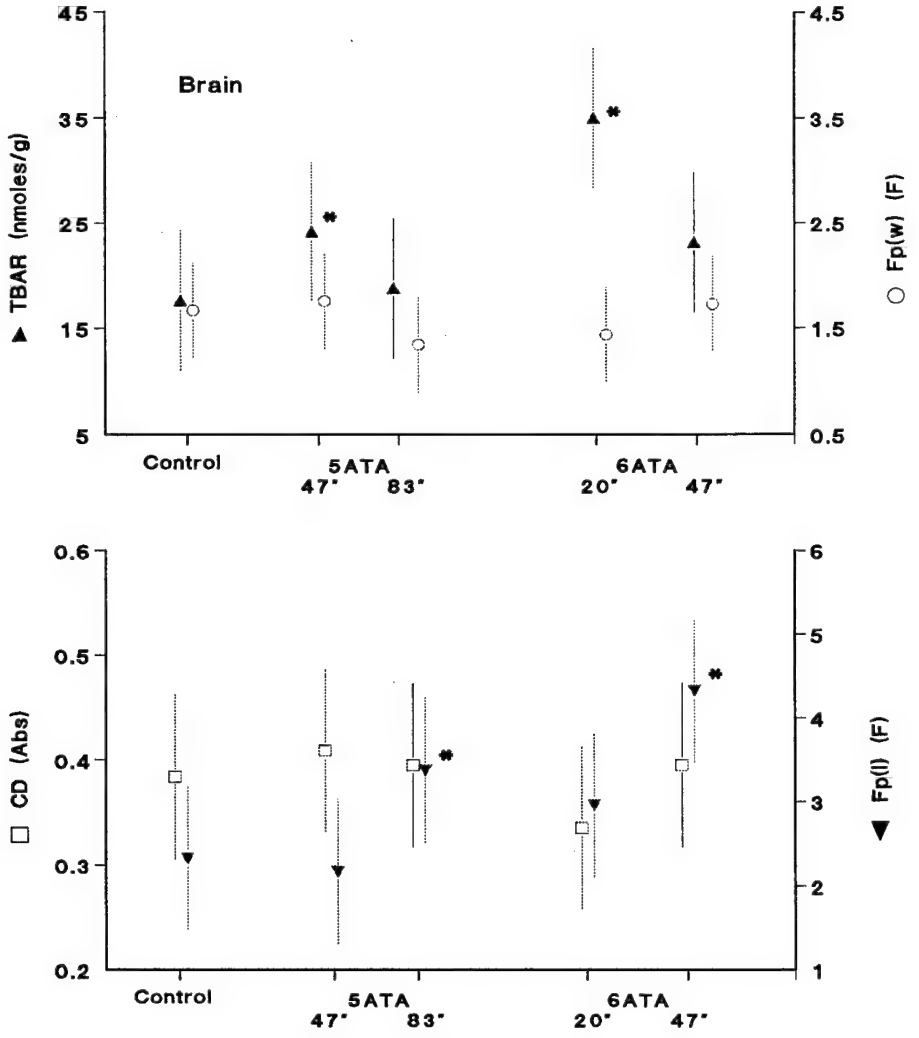

Figure 7. Effect of *in vivo* hyperbaric O_2 exposure on 4 indices of lipid peroxidation in rat brain. Animals were exposed to 5 or 6 ATA until development of a convulsion (shorter duration exposures) or until breathing ceased (longer duration). Results are from at least 6 animals in each group, mean ± 1 SD. * Different from control, $P < 0.05$. Abbreviations as in Fig 2.

Figure 8 displays the results for **TBAR** in plasma obtained from animals exposed to normobaric and hyperbaric O_2. (No plasma was obtained from animals given long, terminal hyperbaric exposures.) Increased **TBAR** appeared after 6 h of normobaric 100% O_2 and remained elevated until the final day of survival. The 5-day 85% O_2 exposure also resulted in increased plasma **TBAR**. No significant changes were seen in other variables except for decreases in plasma lipid concentration, **CD**, and **Fp(l)** (data not shown), following long normobaric exposures, which were probably due to the fact that animals refuse food and water after about 48 h of O_2 exposure.

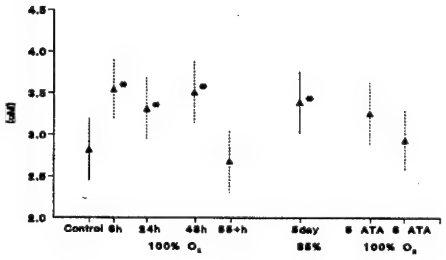

Figure 8. Effect of *in vivo* normobaric and hyperbaric O_2 exposures on **TBAR** in rat plasma. Samples were collected from animals sacrificed after 6, 24, 48, or 55^+ h normobaric 100% O_2, 5 days of 85% O_2, 5 ATA for 47 min or 6 ATA for 20 min. * Different from control, $P < 0.05$. Abbreviations as in Fig. 2.

DISCUSSION

This study compared the progression of 4 indices of lipid peroxidation in isolated tissues and whole animals exposed to a range of O_2 levels. *In vitro*, all 4 markers along the cascade progressively increased with O_2 exposure level in brain and lung homogenates; *in vivo*, there were also indications of a dose-response relationship, e.g. hyperbaric exposures resulted in higher values than the longest normobaric ones, but there was no consistently clear progression in any of the four indices studied. With the exception of plasma **TBAR**, these did not show promise as early markers of oxidative damage. Of particular interest was the observation that brain peroxidation occurred following normobaric O_2 in the intact animal and that plasma **TBAR** showed an early increase that was sustained. Each of these points will be discussed.

In vitro vs. in vivo

The difference between brain and lung **TBAR** formation following *in vitro* exposure was striking. Homogenized brain tissue produced over 80x control levels of **TBAR**, while lung produced about 2x control (Figs. 2 and 3). Plasma produced the smallest increases. Other workers noticing differences in *in vitro* **TBAR** production among tissues tried and failed to account for the differences by lipid (15, 16), antioxidant (17) or iron (18) content. It is interesting that we did not see differences of this magnitude in the other indices measured: **Fp(l)** increased 5-10x over control and **CD** and **Fp(w)** only about doubled in both tissues. In contrast, following whole animal *in vivo* exposures, smaller increments in lipid peroxidation occurred, although these exposures produced clear evidence of pulmonary and central nervous system toxicity. The largest increase in any lipid peroxide variable noted was about 2x

control (Figs. 4-7). Several groups (15, 19, 17), showed that peroxidation potential increased with increasing fractionation procedures (i.e., slices < homogenates < microsomes). Intact animals have abundant and varied antioxidants, and compartmentalization of catalytic metal ions (4) that must minimize both initiation and propagation of lipid peroxidation processes. Our results support the notion that intact animals are more resistant to lipid peroxidation than ground up or sliced tissues.

Dose-response relationship between PO_2 and lipid peroxidation

Although hyperbaric exposures resulted in higher levels of lipid peroxides than the longest normobaric ones, we did not see consistent increases as a function of time and depth. For example, long hyperbaric exposures did not lead to higher concentrations than short ones (e.g., Fig. 5). Also, with the exception of plasma **TBAR** (Fig. 8), (and a few other instances at the 6-hour time point, which will be discussed below) these variables did not increase until after fairly long or high PO_2 exposures. This is most clearly shown with the normobaric results (Figs. 4 and 6) where lipid peroxidation did not occur until the nearly terminal 55^+ h time point. With hyperbaric conditions, exposures long enough to produce lung damage and convulsions did not consistently raise lung (Fig. 5) or brain (Fig. 7) markers of lipid peroxidation.

Choice of index

Caution concerning the use of **TBAR** alone as a marker of lipid peroxidation has been advised (3, 4) for several reasons. Malondialdehyde, the major component of **TBAR,** forms only from lipids containing ≥ 3 double bonds, only a portion of all unsaturated lipids (3). Furthermore, aldehydes are reactive molecules, making them hard to accurately estimate *in*

vivo. Also, there are enzymes to metabolize them such as the mitochondrial enzyme, aldehyde dehydrogenase (4). The boiling acidic conditions of the assay itself encourage formation of malondialdehyde (3). We chose to try to minimize the formation during the assay by using an iron chelator and a chain breaking antioxidant (3). This has not been the approach taken in all studies (1, 6, 7, 20-22) and higher levels will result without these precautions.

Some studies have claimed an advantage of **CD** over **TBAR** (7). Others advise measuring more than one index (3, 4), and this was the approach we chose. Other *in vitro* studies have shown clear temporal relationships between **TBAR** and fluorescent products (5, 23) reflecting the cascade outlined in Fig. 1. No temporal progression from **TBAR** to fluorescent products was apparent in our *in vitro* studies, but results obtained in brain (Fig. 7) could be explained on this basis. Following short hyperbaric exposures, **TBAR** increased but this increase was not maintained following long exposures when **Fp(l)** was elevated. It is possible that the **TBAR** had cross-linked to form an organic fluorescent product (5) in the brain following the longest hyperbaric exposures.

None of the markers studies appears to be ideal. It was rare when one of the others increased and **TBAR** did not. Plasma **CD** and **Fp(l)** are affected by lipid concentration, so normalization is often required, as has also been pointed out by Demling and coworkers (6). We found that plasma lipid concentration decreased during long normobaric exposures, probably due to the animals' anorexia. On the other hand, **TBAR** is a simple assay that is not sensitive to lipid concentration; it is, however, sensitive to hemolysis. This has been shown clearly for the spectrophotometric assay (22). We also found evidence of blood interference

in the fluorescent assay we used. Recovery of added TMP decreased in O_2 exposed organs (described in results). Quenching of **TBAR** due to blood is a serious concern when working with O_2-exposed animals. Blood content of tissues increases with O_2 exposure, even when organs are perfused (14). Furthermore, as an animal is exposed to O_2, it becomes more difficult to obtain non-hemolyzed samples from catheters, probably due to hemoconcentration and perhaps increased fragility of red cells.

Non-pulmonary lipid oxidation with normobaric O_2

We found evidence of oxidative stress in a non-pulmonary organ with normobaric O_2 exposure, namely **TBAR** formation in brain tissue following long exposures to 85 or 100% O_2 (Fig. 5). Crapo and Tierney (9) also saw induction of superoxide dismutase in brain as well as lung in rats exposed to 85% O_2 in their original description of this tolerance-inducing dose. Our results contribute to the accumulating evidence that there are systemic effects, even with normobaric O_2. Although there are clear pulmonary effects and terminal decrements in gas exchange, it is probably not purely respiratory limitation which kills animals (24).

Lipid peroxidation as an early marker

Plasma **TBAR** increased after 6 h of exposure to normobaric O_2 and remained elevated, although it did not increase further with time. The decrease at 55^+ h may have been due to hemolysis, as addressed above, and furthermore, small amounts of hemolysis may have prevented our seeing a progressive rise in plasma levels with time of exposures. The observed early increase in plasma **TBAR** deserves more careful study. Sequential samples drawn from animals would be more sensitive and accurate than the procedure used here. Demling and coworkers (6) have shown with endotoxin-induced lung damage, another model

of oxidative damage, that plasma **TBAR** reflected pulmonary changes.

In addition to changes in plasma, we also saw transient increases in tissue markers after 6 h of normobaric O_2 (Figs. 4 and 6). We chose to look at the 6 h time point because of some reports (25) that expired pentane, a non-invasive indicator of lipid peroxidation, increased in about this time frame. The significance of this transient increase is not clear.

The idea that lipid peroxidation is the initiator in many pathophysiological processes has been challenged by Halliwell (4), who suggests instead that it is a final pathway. Newer studies of oxidation *in vitro* also suggest that protein oxidation may precede lipid peroxidation (26). Our results support these notions and careful studies of changes in protein oxidation following O_2 exposure would be warranted.

To our knowledge this is the only report in which ranges of O_2 exposures, organs, and markers have been simultaneously interrogated. Our work is consistent with evidence showing that lung **TBAR** increases with normobaric exposures (27), with the 85% 5-day exposure (21), and in brain tissue after hyperbaric exposures of fixed length (28-30). More recently, a carefully conducted study by Jamieson (22) failed to find **TBAR** or **Fp(l)** in lung or brain of mice exposed to between 4.5 and 5.9 ATA of O_2 for 30 min. This discrepancy in results is unlikely to be due to species difference. Dirks and Faiman (20) have shown that mice brain slices produce significantly more **TBAR** than rat slices following *in vitro* exposure to a range of hyperbaric O_2. Jamieson's (22) exposures were shorter than ours, but they were of sufficient duration to produce clear symptoms of O_2 toxicity in the mice. Jamieson (22) did not quick freeze the tissues before analysis and our results show that omission of this step can lead to changes. Probably most importantly, however, is the result

of our study, which warns against measurement of only one index at one time point. Our study shows that different time points in a hyperbaric exposure can lead to different increments in different indices.

SUMMARY

Four different indices marking the lipid peroxidation cascade were studied in isolated tissues and in whole animals exposed to normobaric and hyperbaric O_2. No matter which index was used, *in vivo* lipid peroxidation is less dramatic than *in vitro*. In tissues, fairly long duration and/or high PO_2 exposures were required for changes, lending support to the notion that lipid peroxidation may be a consequence of other damage. Lipid peroxide levels in plasma appear to offer promise as an early marker of ongoing changes.

REFERENCES

1. Wood, J.D. and Watson, W.J., "Lipid peroxidation (thiobarbituric acid reactive material) and enzyme inhibition in rat brain homogenates exposed to O_2 at high pressure." Canadian Journal of Physiology and Pharmacology, Vol. 45, pp. 752-755, 1967.

2. Girotti, A.W. and Thomas, J.P., "Damaging effects of O_2 radicals on resealed erythrocyte ghosts." Journal of Biology and Chemistry, Vol. 259, pp. 1744-1752, 1984.

3. Buege, J.A. and Aust, S.D., "Microsomal lipid peroxidation." Methods in Enzymology, Vol. 52, pp. 302-310, 1978.

4. Halliwell, B. and Gutteridge, J.M.C., "Lipid peroxidation: a radical chain reaction." Free Radicals in Biology and Medicine, Oxford Press, Oxford, United Kingdom, Chapter 4, pp. 139-189, 1985.

5. Bidlack, W.R. and Tappel, A.L., "Fluorescent products of phospholipids during lipid peroxidation." Lipids, Vol. 8, pp. 203-207, 1973.

6. Demling, R.H., Lalonde, C., Ryan, P., Zhu, D. and Liu,Y., "Endotoxemia produces an increase in arterial but not venous lipid peroxides in sheep." Journal of Applied Physiology, Vol. 64, pp. 592-598, 1988.

7. Ward, P.A.,Till, G.O., Hatherill, J.R., Annesley, T.M., and Kunkel, R.G. "Systemic complement activation, lung injury, and products of lipid peroxidation." Journal of Clinical Investigation, Vol. 76, pp. 517-527, 1985.

8. Rehncrona, S., Smith, D.S., Akesson, B., Westerberg, E. and Siesjo, B.K. "Peroxidative changes in brain cortical fatty acids and phospholipids as characterized during Fe^{2+} and ascorbic acid stimulated lipid peroxidation *in vitro*." Journal of Neurochemistry, Vol.

34, pp. 1630-1638, 1980.

9. Crapo, J.D. and Tierney, D.F., "Superoxide dismutase and pulmonary oxygen toxicity." American Journal of Physiology, Vol. 226, pp. 1401-1407, 1974.

10. Ohkawa, H., Ohishi, N., and Yagi, K. "Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction." Analytical Biochemistry, Vol. 95, pp. 351-358, 1979.

11. Takeda, K., Shimada, Y., Amono, M., Sakai, T., Okada, T., and Yoshiya, I. "Plasma lipid peroxides and alpha-tocopherol in critically ill patients." Critical Care Medicine, Vol. 12, pp. 957-959, 1984.

12. Bligh, E.G. and Dyer,W.J., "A rapid method of total lipid extraction and purification." Canadian Journal of Biochemistry and Physiology, Vol. 37, pp. 911-917, 1991.

13. Chiang, S.P., Gessert, C.F., and Lowry, O.H., Colorimetric determination of extracted lipids. USAF Texas Research Report 56-13, Air University School of Aviation Medicine, Texas, June 1957.

14. Harabin, A.L., Braisted, J.C. and Flynn, E.T. "Response of antioxidant enzymes to intermittent and continuous hyperbaric oxygen." Journal of Applied Physiology, Vol. 69, pp. 328-335, 1990.

15. Cadenas, E., Varsavsky, A.I., Boveris, A., and Chance, B., "O_2 or organic hydroperoxide induced chemiluminescence of brain and liver homogenates." Biochemistry Journal, Vol. 198, pp. 645-654, 1981.

16. Franco, D.P. and Jenkinson, S.G., "Rat lung microsomal lipid peroxidation: effects of vitamin E and reduced glutathione." Journal of Applied Physiology, Vol. 61, pp. 785-790, 1986.

17. Kornbrust, D.J. and Mavis, R.D. "Relative susceptibility of microsomes from lung, heart, liver, kidney, brain and testes to lipid peroxidation: correlation with vitamin E content." Lipids, Vol. 15, pp. 315-322, 1980.

18. Zaleska, M.M. and Floyd, R.A. "Regional lipid peroxidation in rat brain in vitro: possible role of endogenous iron." Neurochemical Research, Vol. 10, pp. 397-410, 1985.

19. Fraga, C.G., Leibovitz, B.E., and Tappel, A.L., "Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: characterization and comparison with homogenates and microsomes." Free Radical Biology and Medicine, Vol. 4, pp. 155-161, 1988.

20. Dirks, R.C., and Faiman, M.D., "Free radical formation and lipid peroxidation in rat and mouse cerebral cortex slices exposed to high O_2 pressure." Brain Research, Vol. 248, pp. 355-360, 1982.

21. Freeman, B.A., Topolsky, M. K., and Crapo, J.D., "Hyperoxia increases oxygen radical production in rat lung honmogenates." Archives of Biochemisty and Biophysics, Vol. 216, pp. 477-484, 1982.

22. Jamieson, D.D., "Lipid peroxidation in brain and lungs from mice exposed to hyperoxia." Biochemical Pharmacology, Vol. 41, pp. 749-756, 1991.

23. Noll, T., DeGroot, H., and Sies, H. "Distinct temporal relationship among O_2 uptake, malondialdehyde formation, and low-level chemiluminescence during microsomal lipid peroxidation." Archives of Biochemistry and Biophphysics,. Vol. 252, pp. 284-191, 1987.

24. Harabin, A.L., Homer, L.D. and Flynn, E.T., "Pulmonary oxygen toxicity in awake dogs: metabolic and physiological effect." Journal of Applied Physiology, Vol. 57, pp. 1480-1488, 1984.

25. Habib, M.P., Eskelson, C., and Katz, M.A., "Ethane production rate in rats exposed to high concentrations of oxygen." American Review of Respiratory Diseases, Vol. 137, pp. 341-344, 1988.

26. Davies, K.J.A. and Goldberg, A.L., "O_2 radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes." Journal of Biology and Chemistry, Vol. 262, pp. 8220-8226, 1987.

27. Mansour, H., Levacher, M., Azoulay-Dupuis, E., Moureau, J., Marquetty, C., and Gougerot-Pocidalo, M. "Genetic differences in response to pulmonary cytochrome *P*-450 inducers and O_2 toxicity." Journal of Applied Physiology, Vol. 64, pp. 1376-1381, 1988.

28. Jerrett, S.A., Jefferson, D., Mengel, C.E. "Seizures, H_2O_2 formation and lipid peroxides in brain during exposure to oxygen undr high pressure." Aerospace Medicine, Vol. 44, pp. 40-44, 1973.

29. Noda, Y., McGeer, P.L., and McGeer, E.G. "Lipid peroxide distribution in brain and the effect of hyperbaric oxygen." Journal of Neurochemistry, Vol. 40, pp. 1329-1332, 1983.

30. Zirkle, L.G., Mengel, C.E., Horton, B.D., Duffy, E.J. "Studies of oxygen toxicity in the central nervous system." Aerospace Medicine, Vol. 36, pp. 1027-1032, 1965.